Journal of Invertebrate Pathology 104 (2010) 58–65

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Carbon utilization pattern as a potential quality control criterion for virulence of *Beauveria brongniartii*

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ARTICLE INFO

Article history:
Received 27 August 2009
Accepted 27 January 2010
Available online 1 February 2010

Keywords:
Beauveria brongniartii
Entomopathogenic fungus
Biological control agent
Carbon utilization
Virulence indicator
Microtiter plate system
Quality control

ABSTRACT

The registered entomopathogenic fungus *Beauveria brongniartii* (BIPESCO 2) was tested for its virulence after one, five and 10 times sub-culturing on four types of selective synthetic nutrient media. Bioassays with third instar *Melolontha melolontha* larvae showed that sub-culturing negatively affects the virulence of the fungus after 10 transfers. With the Biolog™ SF-P2 and Biolog™ SF-N2 microtiter plate systems the sub-cultivated *B. brongniartii* conidia were monitored for any change in the carbon utilization pattern of 128 carbon sources. With the help of Spearman's rank correlation, principal components analysis and canonical correspondence analysis, respectively, six carbon sources were identified as potential virulence indicators for BIPESCO 2 (pyruvic acid, maltose, glycyl-L-glutamic acid, malonic acid, glucuronamide and phenylethylamine). The Biolog™ microtiter plate system is suggested as a simple and inexpensive test-system for virulence determination of *B. brongniartii* strain BIPESCO 2 in routine quality control.

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1. Introduction

Efficacy, safety and stability are key issues which define an effective biocontrol agent (BCA). In the case of insect–pathogenic fungi, the efficacy of products requires the utilization of highly virulent strains, which also persist to a certain extent in the environment after application (Butt, 2002; Zimmermann, 2007).

Producers of BCAs are not only requested to improve their products for marketing reasons, they also have to develop methods and tools – preferably as standard protocols – to address key issues for quality control (European Commission, 2001). It is in their interest that quality control methods are simple, inexpensive and time-saving, and particularly refer to the virulence of the BCAs.

The maintenance of virulence is crucial, as strains tend to attenuate in the course of successive sub-culturing on laboratory media, or by storing in refrigerator systems or liquid nitrogen (MacLeod, 1954; Morrow et al., 1989; Coremans-Pelseneer and Tillemans, 1991; Butt et al., 2006). Strains of *Beauveria brongniartii*, a highly virulent fungus, pathogenic against the scarabs *Melolontha melolontha* and *M. hippocastani*, were demonstrated to attenuate in virulence as quickly as after the sixth successive sub-cultivation (Pernfuss, 2002; Strasser and Pernfuss, 2005). For Melocont™-Pilzgerste production strain *B. brongniartii* – BIPESCO 2 – a decline in virulence was observed within passage eight to passage 14 if cultivated on Sabouraud-2% glucose agar.

It is still unclear which characteristics of the fungi are changed exactly when a decline in virulence is observed. Up to now the sole possibility to overcome that phenomenon is a periodical passage via the target host (Butt and Goettel, 2000). But this procedure is very time-consuming and there is a significant danger of contamination. For that reason, a fast and reliable virulence check is needed to indicate the time for this critical re-freshing procedure. As laboratory bioassays against *Melolontha* spp. require several weeks in the case of *B. brongniartii* and standard enzyme tests (e.g. for pr1-activity) have led to contradicting results for strain BIPESCO 2 (unpublished results), we studied carbon utilization patterns of highly virulent and moderately virulent states of the *B. brongniartii* production strain. Such carbon utilization patterns were earlier applied for differentiation of *Beauveria* strains and species data suggested that they are linked to virulence (Pernfuss, 2002; Pernfuss et al., 2003). Starting from a single-cell culture of *B. brongniartii*, serial sub-cultivations on four selective cultivation media were performed to enforce fungal attenuation and to monitor any possible change in the ability to metabolise 128 carbon sources. Sophisticated statistical analyses were performed to find correlations between the decrease in virulence and the utilization of particular carbon sources.

2. Materials and methods

2.1. Strain

Different virulence states of *B. brongniartii* (Sacch.) Petch, strain BIPESCO 2 (CBS 110631) originally isolated from *M. melolontha* (L.) infested soil in Kramsach (Tyrol, Austria) were used. The highly

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0022-2011/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2010.01.007

virulent production strain was sub-cultivated up to 10 times on different media (see below) to achieve moderately virulent (attenuated) phenotypes.

2.2. Cultivation

Strain BIPESCO 2 was grown on four media: (i) Sabouraud-2% glucose agar (S2G), (ii) complete agar medium (CAM, Tarocco et al., 2005), (iii) modified complete agar medium (CAM_m, yeast extract substituted by trace metals (in mg L^{-1}: 400 $CaCl_2 \cdot 2H_2O$, 50 $FeSO_4 \cdot 7H_2O$, 16 $MnSO_4 \cdot H_2O$, 14 $ZnSO_4 \cdot 7H_2O$) and vitamins (500 µg L^{-1}: nicotinic acid, pantothenate, pyridoxamine dichloride, riboflavin, thiamine dichloride; 50 µg L^{-1}: biotin, cyanocobalamine, folic acid)), and (iv) complete agar medium containing the target host *M. melolontha* (CAM_{HM}, yeast extract substituted by 1% (w/v) insect homogenate).

Sub-cultivations were done by inoculation of the plates with 50 µL of pure conidial suspensions (1×10^5–2×10^8 conidia mL^{-1}) prepared from respective media with 0.1% (w/v) Tween® 80 and purified from mycelia fragments by the method of Newmeyer (1990) to ensure whole developmental cycles. The same spore suspensions (with adapted spore concentrations) were used for virulence determination as well as carbon utilization experiments (both after serial cultivation 1, 5 and 10). Cultures were incubated in the dark at 25 °C and RH > 65% for 14 days.

2.3. Determination of virulence

At least 6 weeks-quarantined third instar *M. melolontha* (L.) larvae were subjected to a BIPESCO standard dipping bioassay (Laengle et al., 2005). Larvae were dipped for 5 s into the respective conidial suspension (1×10^7 conidia mL^{-1} in 0.1% (w/v) Tween® 80; $n = 15$) and placed individually into plastic containers. Larvae were kept for 60 days at 20 °C under moist conditions in the dark, fed weekly with a slice of carrot and monitored for mortality at least every fourth day.

Bioassay data was analyzed following the Probit method by Throne et al. (1995) using Mathematica software® 4.2. LT_{50} values were calculated using probit transformation of proportion of insects killed and logarithmic transformation of time. They were used for comparison using one-way ANOVA and correlation analysis (SPSS version 14.0). Analysis of variance was performed to estimate differences among media and sub-cultivations with respect to LT_{50} values. A Spearman's rank correlation was used to estimate a potential correlation between LT_{50} values and number of sub-cultivation.

2.4. Measurement of carbon utilization rates

Carbon source utilization was measured using Biolog™ SF-P2 and Biolog™ SF-N2 microtiter plates, which enable the screening of 128 different carbon sources and can be used for easy and rapid characterization of filamentous microorganisms (Biolog 2009, http://www.biolog.com). The preparation of the microtiter plate test-system was carried out with some modifications of the manufacturer's instruction.

Microtiter plates were inoculated with germlings to exclude any stimulating or repressing influence on conidia germination moderated by certain carbon sources. Accordingly, 1 mL of conidia suspensions from different media origin (4×10^7 conidia mL^{-1}, each) was added to 20 mL of the respective liquid medium (three duplicates each). Flasks were incubated at 25 °C and 180 rpm until more than 70% of conidia were germinated (microscopic evaluation).

Per attempt, 15 mL of two liquid cultures were centrifuged under sterile conditions for 15 min at 15,280*g* at room temperature to separate germinated conidia from nutrient media. Supernatants were decanted under sterile conditions and conidia were re-suspended in 15 mL of sterile bi-distilled water. Tubes were centrifuged again under the same conditions. After decanting the water, conidia were re-suspended in 5 mL of sterile 0.2% (w/v) Carrageenan Type II (Sigma C-1138). The contents of the two duplicates were pooled in one tube and spore concentrations were set to $(2 \pm 0.2) \times 10^5$ germinated conidia mL^{-1} Carrageenan solution. Microtiter plates – three parallels per attempt and plate type – were inoculated with 100 µL spore solution and incubated in a moist chamber at 25 °C in the dark. After 72 h of incubation, optical density of the wells was measured at 590 nm using a Tecan™ Sunrise microplate reader.

Per plate, the optical density of the reference well (containing water and inoculum) was subtracted from optical densities of the wells containing carbon sources. Negative values were set to zero. In order to calculate the relative carbon utilization (RCU), an internal standard was selected which was characterised by similar values in all attempts and triplicates. The value of this internal standard (α-D-glucose) was set to 100% and the relative utilization of the other carbon sources was calculated.

RCU values were used for comparison using one-way ANOVA, correlation analysis, canonical correspondence analysis and principal components analysis. Analysis of variance was conducted to determine differences among media and sub-cultivations in regard to the RCU (SPSS 14.0). Canonical correspondence analysis (CCA) was used to estimate the relationship of RCU with respect to the different sub-cultivations (Canoco 4.53; Ter Braak and Smilauer, 2004). A Spearman's rank correlation was used to find potential correlation between RCU and LT_{50} values (SPSS 14.0). Carbon sources of which utilization showed extremely significant correlations with LT_{50} values ($p \leqslant 0.001$), were used as variables for a principal components analysis (PCA) to estimate differences between virulent and attenuated attempts (Statistica 7.1).

3. Results

3.1. Virulence monitoring

BIPESCO 2 conidia from all media and sub-cultivations 1 and 5 were virulent to the target host *M. melolontha*. Virulence did not significantly differ with respect to cultivation media (Kruskal–Wallis test, $p = 0.932$). None of the media showed obviously improving, stabilizing or limiting effects on the fungal virulence during sub-cultivation. Accordingly, a reduction in virulence was found on all media tested: LT_{50} values obtained with conidia from sub-cultivation 1 did not exceed 50 days representing a high virulence. With progressive sub-cultivation LT_{50} values increased (Spearmen's rank correlation, $p = 0.003$, correlation coefficient = 0.482). Evident rises of LT_{50} (i.e. a significant reduction of virulence) were, however, in most cases only observed after sub-cultivation 10, where LT_{50} values were in general greater than 50 days. Analysis of variance indicated differences among the sub-cultivations with respect to the LT_{50} values (Kruskal–Wallis test, $p = 0.003$): by comparing LT_{50} values stemming from the pathogenic activity of spores from two different sub-cultivations, a significant difference in virulence was confirmed only between sub-cultivations 1 and 10 (Mann–Whitney-U test, $p = 0.003$) and between sub-cultivations 5 and 10 (Mann–Whitney-U test, $p = 0.002$), but not between sub-cultivations 1 and 5 (Mann–Whitney-U test, $p = 0.773$). Thus, a significant attenuation in virulence generally occurred from sub-cultivation 5 to sub-cultivation 10. In some rare occasions, spores from sub-cultivation 10 even seemed to be completely attenuated in virulence by exhibiting calculated LT_{50} values >400 days. Nevertheless, focusing on single sub-cultivation sequences only, there were few cases where virulence was stabilized up to sub-cultivation 10 (all LT_{50} val-

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